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pH-SENSITIVE STRIPS BASED ON CELLULOSE AND ANTHOCYANINS FROM DRIED JAVA PLUM FRUITS (*Syzygium cumini*)

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ABSTRACT

This study aimed to develop pH-sensitive strips made of cellulose incorporated with anthocyanins extracted from dried Java plum fruits (*Syzygium cumini*) which potentially can be used to monitor food freshness. The spoilage of animal protein typically produces total volatile basic nitrogen (TVB-N), which can be easily detected using pH-sensitive indicators. pH-sensitive indicators can be developed by combining biopolymers and anthocyanins. Biopolymers were cellulose from Whatman filter paper. While anthocyanins in this study were extracted from dried Java plum fruits (*Syzygium cumini*) with acidified water (0.01% v/v HCl) followed by SPE. Anthocyanins were incorporated into Whatman filter paper and dried at 70 °C for 10 min. Cellulose incorporated with anthocyanins was analysed using FT-IR and tested for pH 7-10. LC-MS spectra showed cyanidin-3-*O*-glucoside (m/z 449.3), delphinidin-3-*O*-glucoside (m/z 465.3), and petunidin-3-*O*-glucoside (m/z 479.3). These anthocyanins were the products of the deglycosylation of anthocyanin diglycosides. The deglycosylation of anthocyanins takes place through two different routes in either hemiketal or quinonoid forms. Those proposed two pathways are through protonation on an oxygen atom connecting an aglycone and a sugar moiety or through protonation on an oxygen atom within a sugar ring moiety. UV-Vis studies showed the colour profiles of anthocyanins in buffer solutions pH 1-12. The $\lambda_{vis\text{-}max}$ of flavylium at pH < 3 ranged from 515-524 nm. At pH 4 to 6, colourless hemiketal was observed. The $\lambda_{vis\text{-}max}$ of the quinonoid base was 575 nm at pH 7 and 590-599 nm for quinonoid anion at pH > 8. In alkaline pH, chalcone was observed.

Keywords: *pH-sensitive strip, cellulose, anthocyanins, Java plum, Syzygium cumini*

INTRODUCTION

The freshness of foods is one of the customers' primary concerns regarding food safety, as spoiled foods pose a risk of jeopardising human health [1]. Additionally, the foods' nutrients reduce over time. In general, macromolecules such as polysaccharides, cellulose, and proteins have limited antioxidant and antibacterial activities [2]. In general, the freshness of foods can be detected by 1) pH; 2) the formation of diphenyltetrazolium chloride (TTC) as standard mitochondrial redox potential for cell death; and 3) the production of some metabolites, for instance, total volatile nitrogen compounds (TVBN) including ammonia (NH_3), dimethylamine

(DMA, $NH(CH_3)_2$), and trimethylamine (TMA, $N(CH_3)_3$) [3], [4]. TVBN, one of the biochemical markers of meat-based food spoilage, can be identified as follows: low TVBN values indicate that the meat is still fresh and vice versa [5]. In addition, the changes in colour and flavour of food mean food spoilage. However, these compounds are toxic, leading to the mortality of humans who try them. Therefore, to alleviate the problem, new research in detecting food spoilage in an easy, affordable, and no toxic way is still worth investigating.

The customer's awareness of safe foods intrigued many researchers to develop new packaging products or indicators that can detect the spoilage of foods. One of the indicators to detect food spoilage is a pH-sensitive strip. A pH-sensitive strip usually consists of a biomaterial and a dye sensitive to the pH changes, such as anthocyanins or other synthetic pigments [6]. These pH-sensitive strips can show a signal, such as a colorimetric or chemical, responding instantaneously to any changes in the initial conditions and food quality. Zhang *et al*. reported the development of pH-sensitive films based on starch/polyvinyl alcohol incorporated with anthocyanins from purple sweet potatoes to monitor shrimp decomposition [7]. Lee *et al.* developed a three-layer indicator which consists of poly(ether-block-amide) (PEBA) film, eight polymer-immobilised dyes (alizarin red, bromocresol green, bromophenol blue, bromothymol blue, m-cresol purple, cresol red, thymol blue), and poly(ethylene terephthalate) film to monitor chicken breast spoilage [8]. As such, utilisation of anthocyanin-containing biomaterial, especially from new sources such as natural colorant-based pH-sensitive indicators, has tremendous potential for further application of smart packaging of a wide range of protein-based products [9]. However, anthocyanins used in literature did not include SPE to remove some impurities in extracts. As such, impurities might affect the colour of the pH indicator.

Anthocyanins are easily found in fruits, flowers, vegetables, or other parts of plants. These compounds are water soluble and non-toxic and have been reported to display red, orange, blue and purple shades in plants [10]–[12]. The biosynthesis of anthocyanins occurs in the cytoplasm through the general flavonoid pathway. Afterwards, anthocyanins are accumulated and stored in vacuoles [13]. Because of their exciting colour properties, anthocyanins' most application is a natural colorant in foods, beverages, textiles, and smart packaging [14]. The attractive property of anthocyanins is that these compounds can dramatically change into various species determined by the pH of the solution, displaying multiple colours. The most substantial form of anthocyanins is red cationic flavylium in an acidic solution (pH < 3). At greater pH, anthocyanins are also present as a hemiketal (**B**), quinonoid base (**A**), and chalcone forms ($\mathbf{C_E/C_Z}$) via competition of two reaction routes (kinetic and thermodynamic) [15], [16]**.** The ability of anthocyanins to transform into various species inspired to make an indicator in detecting food spoilage.

One of the sources of anthocyanins that are worth exploring is Java plum fruits

(*Syzygium cumini*). The major anthocyanins found in this fruit are delphinidin-, petunidin-, and malvidin-3,5-*O*-diglucoside [17]. Some monoglycosides of anthocyanins, such as cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside are also present [18]. The fruit skins contain higher anthocyanins than in fruits or juices due to the plant's system defending itself from UV radiation, protecting the seeds [19]. Importantly, as some consider Java plum fruits "rarely worth eating" due to their astringency, they do not compete with land for food use, which is a vital issue in sustainable and renewable areas. Applying anthocyanins from Java plum fruits in pH-sensitive strips could also add value to this fruit. Therefore, the development of accessible, cheap, fast, and reliable indicators for monitoring the freshness of the products was studied in this research. Increased level of TVBN during protein-based food spoilage successively alters the environment inside the package towards alkaline conditions, which can be detected through colorimetric sensing indicators [4]. Initially, the pH-sensitive strips based on cellulose from Whatman filter paper and anthocyanins from Java plum fruit extract were developed and utilised to measure the pH of buffer solutions at pH 7-10, as at these alkaline pHs where the spoilage of foods is usually detected.

METHODS

Chemicals and Materials

Java plum fruits (*Syzygium cumini*) were bought from the local market and were dried in sunlight for two days. In this study, all solvents and reagents were acquired from Merck (USA) and Sigma Aldrich (USA), where the solvents were in high-performance liquid chromatography (HPLC) and analytical grade. Those solvents were utilised without additional purification. Amberlite XAD-7 HP resin was purchased from Sigma Aldrich (USA) and used during the solid-phase extraction (SPE). Cellulose-based strips were made from Whatman filter paper with a thickness of 210 µm.

Extraction of anthocyanins from dried Java plum fruits

Extraction and purification of anthocyanins from dried Java plum fruits were conducted according to the previous method [14]. Dried Java plum fruits (50 g) were extracted with acidified water (0.01% v/v HCl) using a solid-to-solvent ratio of 1:20 (w/v), at 60 °C, for 3h. The crude extract was filtered using Whatman filter paper through gravity filtration. Afterwards, it was cooled to room temperature and poured slowly into an SPE column packed with Amberlite XAD-7 HP resin. The SPE resin loaded with the Java plum fruit crude extract was washed with acidified water (0.01% v/v, hydrochloric acid; 500 mL) followed by ethyl acetate (250 mL) and acidified ethanol (0.01% v/v, hydrochloric acid; 250 mL). The anthocyanin-containing ethanol was then evaporated under reduced pressure on a rotary evaporator at 40 °C. The resulting anthocyanin-rich extract was then stored in a fridge until further use. For analysis by LC-MS spectroscopy, the anthocyanin-rich extract was dissolved in acidified (0.1% v/v, hydrochloric acid) water: ethanol with a ratio of 9:1 (v/v) [20].

Colour profiles of anthocyanins

To study the colour profiles of anthocyanins, extracts from dried Java plum fruits (*Syzygium cumini*) were added with acidified (0.1% v/v HCl) water: ethanol using a ratio of 9:1 (v/v) and labelled as stock solutions. The stock solution of anthocyanin was then added to buffer solutions with varying pH from 1-12. The colour changes were observed and identified using a double-beam UV-Vis spectrophotometer UV 1800. In addition, the maximum wavelength of each buffer solution was determined.

Preparation and characterisation of pH-sensitive strips

Anthocyanin-containing extracts from dried Java plum fruits were diluted in ethanol. The Whatman filter papers (2 cm x 5 cm) were soaked in anthocyanin solutions and dried in the oven at 70 °C for 10 minutes. The pH-sensitive strips were then analysed using Agilent ATR FTIR (scanning at 650 cm^{-1} to 4000 cm^{-1}) to observe the changes in the functional group of cellulose. The blank Whatman paper was also analysed for comparison. Finally, the dried incorporated cellulose strips with anthocyanins were added with buffer pH 7-10. The colour changes during the addition of buffer solutions were recorded.

RESULTS AND DISCUSSION

In this study, anthocyanins from dried Java plum fruits (*Syzygium cumini*) were extracted, purified, and identified. The colour profile of anthocyanins with different pH was also assessed. Due to the colour properties displayed by anthocyanins, these compounds were incorporated into cellulose (filter paper) and used as pH-sensitive strips. This application is beneficial for monitoring the freshness of products with high content of proteins, such as shrimp, fish, meats etc., as these products are prone to decomposition, endangering customer safety. The decomposition of proteins produces amine derivatives which are alkaline (pH > 7) and could be detected by anthocyanins through colour changes. Anthocyanins at alkaline pH form quinonoid base (**A**), quinonoid anion ($\mathbf{A^-}$), and chalcone ($\mathbf{C_E/C_Z}$). Therefore, a preliminary study on the field of pH-sensitive smart packaging based on cellulose and anthocyanins from Java plum fruits was conducted. First, the FTIR spectra of cellulose and cellulose incorporated with anthocyanins are compared. Afterwards, the pH-sensitive strips based on cellulose and anthocyanins are tested to buffer solutions pH 7-10, observing the colour changes.

Anthocyanins present in dried Java plum fruits (*Syzygium cumini*)

Dried Java plum fruits were extracted with water, a sustainable solvent, with an addition of 0.01% v/v hydrochloric acid at 60 °C for 3 h. The crude extract was further purified with a solid phase extraction (SPE, biomass: Amberlite XAD-7 ratio of 1:1) and evaporation of anthocyanin-rich ethanolic eluates in accordance with a reported procedure in literature [14]. The profile of anthocyanins was analysed by liquid chromatography–mass spectroscopy (LC-MS). In this study, three main anthocyanins were identified from the dried Java plum extract by LC-MS (**Table 1**). Those anthocyanins were identified as cyanidin-3-*O*-glucoside (**Cy3glc**, m/z 449.3), delphinidin-3-*O*-glucoside (**Dp3glc**, m/z

465.3), and petunidin-3-*O*-glucodise (**Pt3glc**, m/z 479.3). Lestario *et al*. and Tavares *et al*. reported that the main anthocyanins present in *Syzygium cumini* were identified as cyanidin-3,5-*O*-diglucoside (**Cy3,5diglc**, m/z 611), delphindin-3,5-*O*-diglucoside (**Dp3,5diglc**, m/z 627), malvidin-3,5-*O*-diglucoside (**Mv3,5diglc**, m/z 655), petunidin-3,5-*O*-diglucoside (Pt**3,5diglc**,m/z 641), peonidin-3,5-*O*-diglucoside (**Pn3,5diglc**, m/z 625), alongside three anthocyanins identified in this study [18], [21]. However, both peonidin and malvidin monoglycosides and diglycosides were not identified in this study. It has been reported that the fact that plant variety, weather, nutrients, soils, and extraction procedures were different, causing different anthocyanin profiles. Additionally, this discrepancy might occur because of the deglycosylation of anthocyanins during the storage, as the excess of strong acid in the extract could lead to the removal of the sugar moiety. In this study, anthocyanin extracts were stored in the fridge. Therefore, it is suggested that deglycosylation might occur because of the presence of strong acid in the extract.

Table 1. Anthocyanins in Java plum fruit

Experimental	**Theoretical** [18], [21]	
[M⁺] m/z	**[M⁺] m/z**	**Anthocyanin**
465.3	465	Dp3glc
479.3	479	Pt3glc
449.3	449	Cy3glc

Anthocyanin colour profiles at pH 1-12

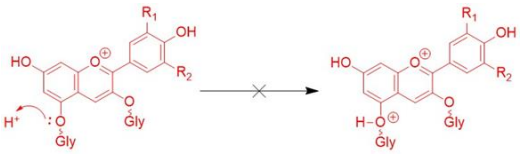
Colour profiles of anthocyanins from dried Java plum fruit extracts at buffer solutions pH 1 to 12 were measured with a UV-Vis spectrophotometer at 200-800 nm. The maximum absorbance of anthocyanins at each pH was recorded and summarised in **Table 2**. The maximum absorbance was used to determine the maximum wavelength. The respective observed colour shows the major anthocyanin species present in the solution. At acidic pH (pH < 3), anthocyanins are predominantly found in the red flavylium cationic form with a maximum visible wavelength ranging from 515-524 nm. At pH 4 to 6, colourless hemiketal forms dominate. This finding is in agreement with the data reported by Basílio and Pina [22]. Meanwhile, a purple quinonoid base was found at 575 nm at pH 7. In alkaline pH (pH > 8), anthocyanins are present as a blue quinonoid anion, which shows a maximum visible wavelength at 590-599 nm. Increasing the pH will transform anthocyanins into yellow chalcone [23]. The solution turned green at pH 10 and 11, indicating that the blue anionic quinonoid and chalcone were in equilibrium. These colour profiles of anthocyanins can be used as a pH indicator, showing different colour changes at different pHs.

Table 2. Anthocyanin form at each pH, including the colour observed and $\lambda_{vis\text{-}max}$ (nm) at pH 1-12.

pH	**Colour observed**	**$\lambda_{vis\text{-}max}$ (nm)**	**Anthocyanin form**
1	Red	515	Flavylium cation
2	Red	516	Flavylium cation
3	Red	524	Flavylium cation
4.5	Colourless	-	Hemiketal
5	Colourless	-	Hemiketal
6	Colourless	-	Hemiketal
7	Purple	575	Quinonoid base
8	Blue	590	Quinonoid anion
9	Blue	599	Quinonoid anion
10	Green	600	Quinonoid anion & chalcone
11	Green	600	Quinonoid anion & chalcone
12	Yellow	400	Chalcone

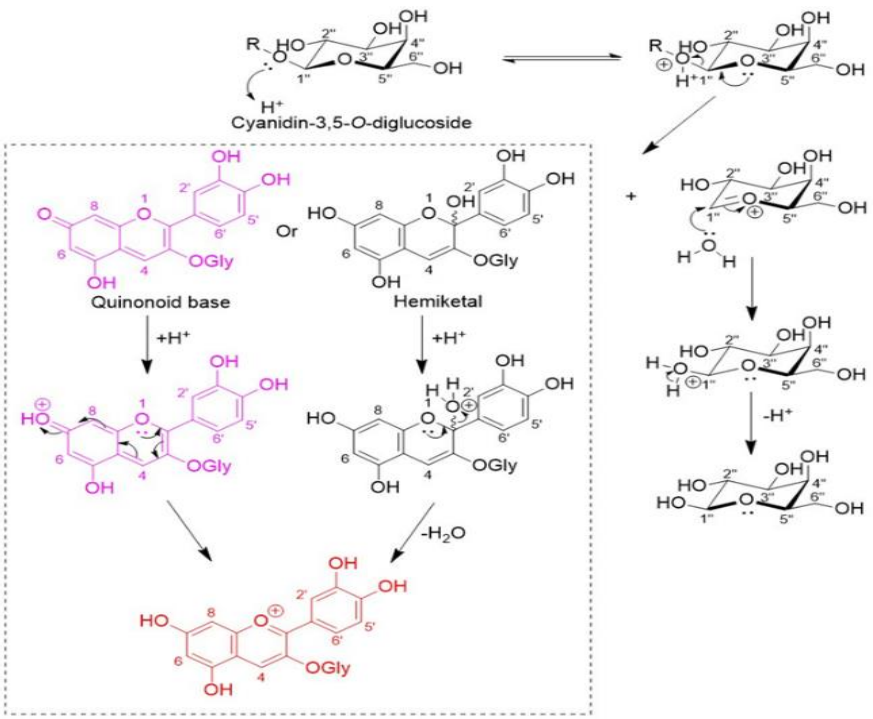
Deglycosylation of anthocyanins (3,5-O-diglucoside) to anthocyanins (3-O-glucoside)

The deglycosylation of anthocyanins with two sugars (3,5-*O*-diglucoside) from dried Java plum fruit extract through a flavylium cationic form is impractical as it is inconceivable to undergo two positively charged oxygen atoms in one molecule (**Scheme 1**). It should be noted that two positive charges in one molecule are generated through the protonation of a flavylium cationic form. Much literature has also reported that a flavylium cationic form is the most stable form amongst other anthocyanin forms at specific conditions [16]. Alternatively, the deglycosylation of anthocyanins (3,5-*O*-diglucoside) from dried Java plum fruit extract might take place through different forms such as the hemiketal form (pH 4-6) or quinonoid base form (pH > 7). The proportion of a flavylium cationic form, hemiketal form, quinonoid form, anionic quinonoid form, and chalcone vary depending on the solution's pH. The first deglycosylation of anthocyanins (3,5-*O*-diglucoside) is proposed to occur on the sugar moiety at position C-5, as in this position, the sugar moiety is less stable than at position C-3 because of steric hindrance. The deglycosylation of anthocyanins can take place through two different pathways, one is through the protonation of an oxygen atom connecting an aglycone and a sugar moiety, and the other is through the protonation of an oxygen atom of sugar ring moiety resulting in a ring-opening in a sugar molecule (**Scheme 2** and **Scheme 3**). However, the sugar will close the ring back at the end of the reaction. This reaction removes one sugar from anthocyanin diglycosides, producing anthocyanins with only one sugar or anthocyanins monoglycosides.



Scheme 1. The proposed general deglycosylation reaction of anthocyanins (3,5-O-diglucoside), forming anthocyanins (3-O-glucoside).

The proposed mechanisms are presented for Cyanidin-3,5-*O*-diglucoside (**Cy3,5diglc**) as an example. Nevertheless, these mechanisms can be applied for the deglycosylation of all anthocyanins with two sugar moieties in general. The presence of H^+ ions and water in the reaction promotes the deglycosylation of anthocyanins, forming anthocyanin with one sugar moiety and unbound sugar molecules. Anthocyanins may be found in hemiketal form or quinonoid base, which will eventually transform into flavylium cationic form. The presence of unbound sugars in the extracts could promote the deglycosylation of anthocyanins due to the hygroscopicity of the sugars, which could attract water. The water promotes the formation of hemiketal, where this compound can further transform into a chalcone. Consequently, anthocyanins start to degrade. The hygroscopicity of anthocyanin standards was reported to range from 10-22% [24].



Scheme 2. Proposed reaction mechanism for the deglycosylation of anthocyanin diglycosides (3,5-*O*-diglucoside). R = Anthocyanin monoglycoside (3-*O*-glucoside) in either hemiketal form or quinonoid base. The protonation takes place at an oxygen atom connecting an aglycone and a sugar moiety at C-5. The products are an anthocyanin monoglycoside (3-*O*-glucoside) in a flavylium cationic form and glucose

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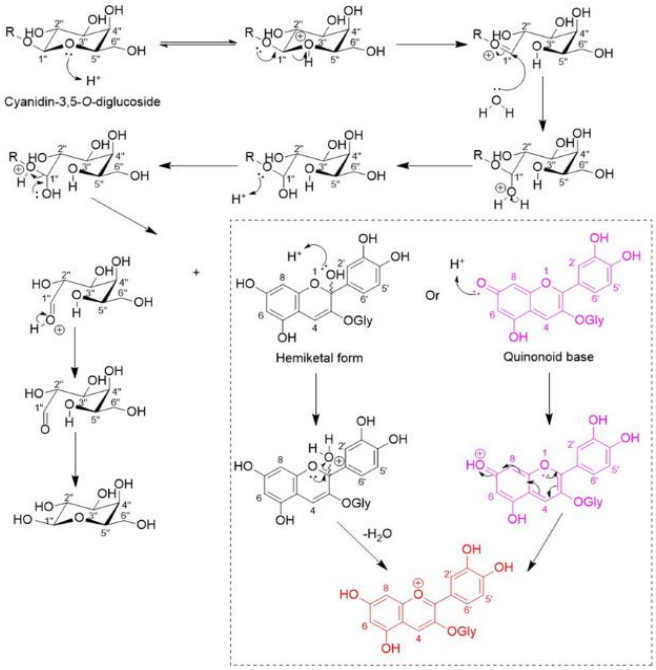

Scheme 3. Proposed reaction mechanism for the deglycosylation of Anthocyanin diglycosides (3,5-*O*-diglucoside). R = Anthocyanin monoglycoside (3-O-glucoside) in either hemiketal form or quinonoid base. The protonation takes place at an oxygen atom in a sugar moiety resulting in a ring opening. The products are an anthocyanin monoglycoside (3-O-glucoside) in a flavylium cationic form and glucose

Application of pH-sensitive strips based on cellulose and anthocyanins from dried Java plum fruits (*Syzygium cumini*)

The acidity of solutions can be identified using a pH meter, litmus paper, or universal indicator paper. In addition, much literature has reported the colour profiles of anthocyanins isolated from various natural sources at different pHs [7], [14], [25], [26]. Because of the anthocyanin's property which shows colour changes at different pHs, one of the applications of anthocyanins is natural colorants, specifically as pH-sensitive strips. pH-sensitive strips can be made from cellulose (Whatman filter paper) and anthocyanins extracted from dried Java plum fruits (*Syzygium cumini*). Cellulose interacts with anthocyanins through hydrogen bonds, as seen in **Figure 1**.

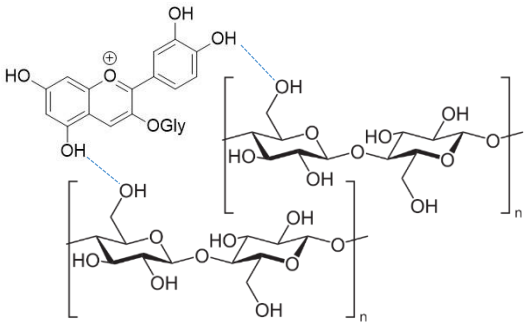

Figure 1. Interaction of cellulose with anthocyanins through hydrogen bonds. Cyanidin-3-*O*-glucoside in flavylium cationic form is given as an example (red). It is noted that anthocyanins at different pHs give different species.

The analysis of samples using Fourier-transform infrared spectroscopy (FT-IR) was conducted to identify whether anthocyanins have been incorporated into cellulose-based strips. Through this technique, infrared radiation is passed through the sample. Some radiations are absorbed, and some are transmitted, producing signals at the detector representing a functional group of organic compounds. The functi onal groups of anthocyanin extracted from dried Java plum fruit were recorded using FT-IR spectroscopy between 650 cm^{-1} to 4000 cm^{-1} at 0.9 cm^{-1} resolution and 48 scans. The comparison of FTIR spectra of cellulose and cellulose incorporated with anthocyanins is presented in **Figure 2**. It must be noted that anthocyanins are incorporated onto cellulose-based strips in a flavylium cationic form, turning the cellulose into the red. A broad absorption band between 3500 and 3200 cm^{-1} indicated a hydrogen bond from hydroxyl (-OH) stretching vibrations. The band at 1374 cm^{-1} was identified as the bending of –OH groups. The –CO stretching occurred at 1198 and 1054 cm^{-1}. The –COC stretching occurred at 1087 cm^{-1}. The –CH bending occurred at 865 and 660 cm^{-1}. The result in this finding is in good agreement with previously reported work where anthocyanins from blueberry were identified using FT-IR [27].

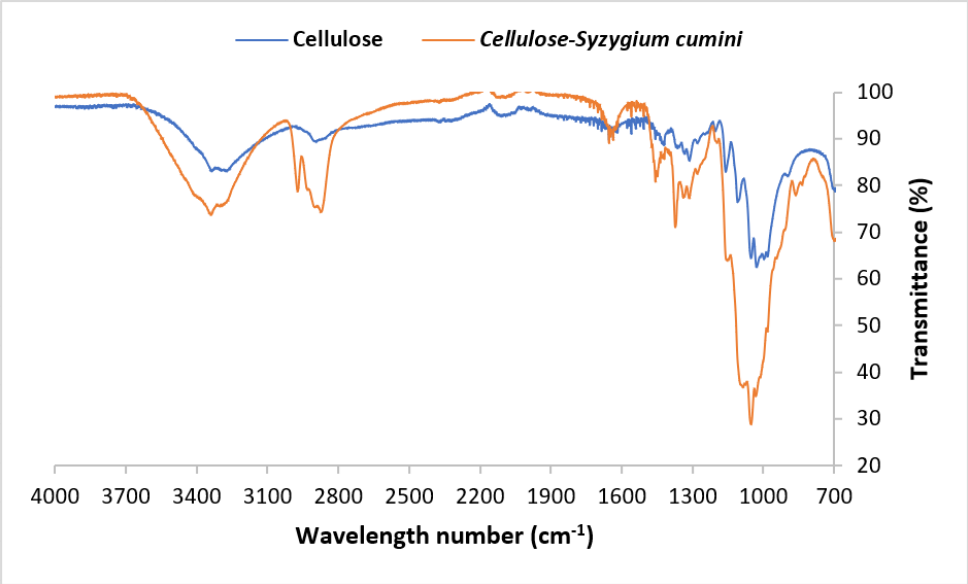

Figure 2. FTIR spectra of cellulose (blue) and cellulose-anthocyanin (orange)

The colour changes of pH-sensitive strips at neutral to alkaline pHs can be seen in **Figure 3**. The initial anthocyanin extract gives the colour red to the cellulose strip, suggesting the flavylium cationic forms dominate and interact with cellulose. At pH = 7, anthocyanins start changing their structures to quinonoid base (**A**), resulting in purple colour. Quinonoid anions can be observed at pH 9, with the colour blue shown in the cellulose strips. In alkaline pH 10 dan 11, anthocyanins are predominantly found as a mixture of quinonoid anion and chalcone, resulting in the colour green as the result of mixing blue and yellow. Usually, the spoilage of foods can be detected by the production of TVBN, which have alkaline properties (pH 9-11). Therefore, pH-sensitive strips made of cellulose and anthocyanins from Java plum fruit can potentially indicate food freshness as they show distinctive colour at different pH. The anthocyanins in this study have been enriched through a SPE. Some impurities were removed, increasing the stability of anthocyanins. This strip is easy to make, affordable, and easily used. The scale-up production and further study are still required to improve the performance of this strip.

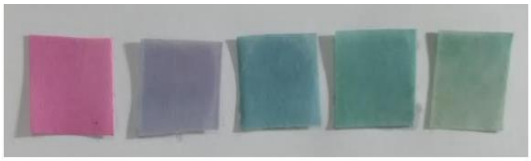

Figure 3. pH-sensitive strips based on cellulose and anthocyanin. After contacting neutral to alkaline pHs, the pH-sensitive strips show different colour

CONCLUSION

Cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, and petunidin-3-*O*-glucodise were identified in the extract of Dried Java plum fruits (*Syzygium cumini*) using LC-MS spectroscopy. Deglycosylation of anthocyanins via hemiketal or quinonoid base forms is possible. The first deglycosylation of anthocyanins (3,5-*O*-diglucoside) occurs on the sugar at position C-5; in this position, the sugar moiety is less

stable than at position C-3. The anthocyanin's deglycosylation can occur through two pathways: protonation of an oxygen atom connecting an aglycone and a sugar moiety, and through protonation of an oxygen atom of sugar ring moiety resulting in a ring-opening in a sugar. At the end of the reaction, the sugar will close the ring back. This reaction removes one sugar, producing anthocyanins with only one sugar. The resultant colour after adding anthocyanins into buffer solutions is due to the transformation of anthocyanins at various pH. It shows the potential application of anthocyanins from Java plum fruit extract as a pH-sensitive indicator to monitor the freshness of foods. However, further study is still required on the effectiveness of this pH-sensitive indicator in detecting food spoilage.

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